APPLICATION FOR RENEWAL OF RESEARCH GRANT

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Project Title: The Mechanism of Increased Cholesterol Uptake by Coronary Arteries (In Vitro Studies)

Background of Proposed Work

As described in our Progress Report, we have now established the fact that human coronary arteries do not synthesize cholesterol, but that cholesterol is transferred directly into the vascular wall from the blood or from the perfusion fluid. In similar studies using saphenous veins, we were able to show that these veins taken from humans during operations also take up cholesterol from the perfusate, the degree of uptake depending on the perfusion pressure. We found that nicotine fails to influence cholesterol uptake or lipid synthesis; finally we established the fact that carbon monoxide leads to a marked increase in cholesterol uptake of human coronary arteries beginning at a concentration of 5% carbon monoxide in the perfusion fluid. Apparently carbon monoxide does not interfere with lipid synthesis in the arterial wall. Our results were obtained on human coronary arteries, since it was discovered that there exist marked species differences as between blood vessels of different species.

The work on carbon monoxide was of particular interest since it demonstrated that carboxyhemoglobin increases the permeability of human coronary arteries to cholesterol. In this respect, CO acts similar to

hypoxia, but its effect is more pronounced. Parving also studied the transvascular protein flux during carbon monoxide exposure and confirmed that the disappearance rate from blood of ^{131}I-albumin increased about 50% after three hours of exposure to 20-25% carboxyhemoglobin. Astrup demonstrated that hypoxia as well as carbon monoxide significantly increase the permeability of endothelial membranes. For example, rabbits, when exposed to carbon monoxide, develop arterial lesions resulting in a considerable accumulation of lipids. They also demonstrated that these animals often accumulated fluid with a high protein content, with a picture of subendothelial edema. Pauli found that carbon monoxide exposure (20-25% carboxyhemoglobin) led to a 50% increase in glomerular filtration rate.

The results as presented in our Progress Report and to be published in the Journal of Atherosclerosis Research agree in general with these animal studies, demonstrating that the permeability of vascular wall of human coronary arteries is altered by carbon monoxide. Although our experiments were conducted in vitro, we interpreted our finding of increased cholesterol uptake under the influence of carbon monoxide as indicating increased permeability of the arterial endothelial layer to cholesterol flux. If one adheres to the concept that cholesterol is actively transported in the vascular wall, our findings imply that biochemical factors controlling the permeability of the arterial wall may be altered by CO. On the other hand, many studies suggest that lipid transport across the arterial wall is a purely passive physico-chemical process. If this is the case, then it could be assumed that CO

modifies those factors which result in an increase in endothelial permeability. As we mentioned in our Progress Report and our publications, no difference in cholesterol uptake was found using two different levels (5 and 90%) of carbon monoxide in the perfusion fluid. This suggests that the rate of cholesterol uptake under these experimental conditions is an all or none process. Furthermore, no influence of carbon monoxide on lipid synthesis in the arterial walls could be demonstrated in our studies.

It is the aim of this investigation to study the mechanism of increased cholesterol uptake under the influence of carbon monoxide using techniques developed in this laboratory. We feel that the principal advantages of our methods are: 1) the experiments are conducted in vitro, so that individual factors can be separate, 2) we can study the effects of various concentrations of carbon monoxide, 3) we can employ arteries from all species, including human coronary arteries; and 4) we can alter at will the hemodynamic conditions with which the vessels are perfused.

Proposed Work

The aim of our proposed investigation is a study of the mechanism of carbon monoxide on cholesterol transfer in perfused coronary arteries.

The study may be divided into two approaches: 1) physico-chemical mechanisms, and 2) the study of hemodynamic causes of increased cholesterol transfer. As indicated in the previous paragraphs, describing the background of our work, carbon monoxide does interfere with the transfer of cholesterol across the arterial wall of human coronary arteries. This

occurs regardless of concentration of carbon monoxide in arterial blood, as long as minimal concentration of from 5-8% is present in the perfusate. The questions to be examined are: a) Can increased permeability be artificially produced? These studies will be carried out using collagenase, an enzyme, which has been shown by Jaffe and his associates (Journal of Clinical Investigation 52:2745, 1973) to lead to digestion of portions of the vascular wall, the degree of change depending on the time to which these vessels are exposed to the enzyme. Thus, for example, it was shown that in specimens examined after collagenase treatment, the endothelial cell lining was electively lost, leaving the basement membrane and underlying structures intact. If, however, collagenase was used in conjunction with manipulation of the vascular wall, the basement membrane was also destroyed.

In a recent series of experiments, the effect of hypoxia on the penetration of cholesterol in the vascular wall will be tested. As mentioned above, there are reasons to believe that the effects of carbon monoxide are at least in part due to hypoxia. For example, Ayres and associates (Archives of Environmental Health 26:8, 1973), demonstrated that the effects of carbon monoxide are primarily related to the leftward shift of the oxygen hemoglobin curve and perhaps also to combinations of carbon monoxide with myoglobin and certain iron-containing enzymes. Hemoglobin-oxygen equilibria in the presence of carboxyhemoglobin resemble the equilibria of more primitive forms of hemoglobin. The oxygen capacity of the blood is decreased, and there

occurs an increase in coronary blood flow and in cardiac output. Britton Chance and Williams demonstrated that a change in respiratory rate produced an alteration in the redox state of the respiratory chain. The relative availability of oxygen, hydrogen-containing substrate, inorganic phosphate and adenosine diphosphate determined whether the chain is more or less oxidized. Since the absorption spectra of respiratory enzymes vary with their redox state, the degree of oxidation may be estimated by spectrophotometric techniques. Therefore, it has been of some concern that carbon monoxide may interfere with the function of all three iron-containing respiratory pigments, hemoglobin, myoglobin and certain cytochromes. This would indicate that CO not only affects permeability of vascular walls, but also cellular respiration directly.

A parallel phase of this investigation will be concerned with the role of hemodynamic factors in the "uptake" or penetration of cholesterol in the arterial wall. This has also been found to be of importance in the mechanism of atherosclerosis.

There are a number of publications dealing with hemodynamic factors in atherogenesis. They are summarized in a paper by Gessner in Circulation Research 33:259, 1973. Several factors are mentioned here which could influence transfer of cholesterol from the blood or the perfusion fluid into the vascular wall. They are the Reynolds number, an actually dimensionless number, which is indicative of the ratio of inertial forces to viscous forces. The Reynolds number can be mathematically defined from the mean velocity averaged over the tube cross-section, the internal diameter of the tube, the density of the fluid, and the absolute viscosity

of the fluid. For viscous flow within a tube, the flow motion near the wall is retarded and a boundary layer developes. In essence therefore we have to examine the laminas to turbulent transitional behavior in a pulsatile flow.

The second factor which may alter vascular permeability to cholesterol is pressure and flow related. It is known that atheromatous plaques appear in regions of low pressure because a suction action exerted on the surface endothelium eventually causes the layers to selectively separate from adjacent tissue. This factor can be easily tested in our in vitro preparations.

The third mechanism exerts itself through wall shear stress. This leads to erosion of the endothelium occurring at sites where the local wall shear stress is relatively high. The theory usually considers simultaneous mechanisms by which arterial wall cholesterol levels may be altered.

Therefore, there must be a series of events which lead to an influx of cholesterol from blood into the vascular wall. There is also the possibility of an opposite movement of cholesterol (from the vascular wall into the blood).

Method of Procedure

The in vitro system of perfusion of human and animal coronary arteries published in three reports from this laboratory will again be utilized; however, both human and dog arteries will be used in these experiments. The reason why we can utilize animal arteries, which synthesize cholesterol, is that one can determine in the control as

well as in the experimental series the ratio of ^{14}C cholesterol/^{3}H cholesterol in arterial walls. By means of this ratio one can derive de novo synthesis of lipids in the arterial wall; ^{3}H cholesterol represents cholesterol moved directly from the perfusate into the arterial wall, ^{14}C uptake represents de novo synthesis. The arteries will be perfused in a modified Carrel-Lindbergh pump at pulsatile pressure. They are perfused at pressures of about 120/80 mmHg, in those experiments in which the effect of collagenase and hypoxia are tested.

In the experiments in which we measure the effect of hemodynamic factors, perfusion and flow rates can be changed at will. Diluted fresh blood, either dog or human blood will be used in the perfusate.

Sterile techniques will be used during the preparation of perfusion, which will be carried out over a period of four hours at 37° centigrade. The gas which drives the fluid through the artery and which will come into the equilibrium with the perfusion fluid consists of 5% CO_2, 25% oxygen and 70% nitrogen. To the perfusion fluid will be added 2-^{14}C sodium acetate and cholesterol, 1, 2-^{3}H. Cholesterol will be added through sonication as described by us in previous reports. It was shown by us that tritium radioactivity is located primarily in the alpha-2-lipoprotein and in the β-lipoprotein fraction of the perfusion fluid. Lipids will be analyzed in the perfusion fluid prior to and following perfusion. Analyses will be carried out on the blood vessels and the extraction of the lipids will be carried out according to the method of Folch. Separation of the lipids will be accomplished by means of thin-layer chromatography on silica gel according to the method of Freeman and West. Radioactivity of the eluate

will be determined in a scintillation vial and counted in a Tri-carb liquid scintillation spectrometer. The method of Zak will be used for the determination of cholesterol. Phospholipids are analyzed according to the method of Lowry modified by Wagner. The blood vessels will be analyzed for lipid synthesis as well as for cholesterol and cholesterol esters uptake for reasons described (to establish the ratio of ^{14}C cholesterol over ^{3}H cholesterol). Statistical analyses will be used to evaluate the results.

In the series where the effect of hypoxia will be studied, one vessel from the same patient or the same animal will be perfused with plasma previously made oxygen free, the other artery will be perfused at normal oxygen saturation and tension. The analytical procedures will be identical to those described above. For the hemodynamic experiments, which will deal with an investigation of the effect of changes in the Reynolds number and pressures and flow, alterations in the Reynolds number will result from partial constriction of the perfused vessel, so as to produce turbulence above and below the vessel. The Reynolds number will de determined as described above.

Pressures and flow can be altered by devices inherent in the mechanism of the pump.

Significance of this Work

Studies presented in the progress report have demonstrated that carbon monoxide in various concentration increases cholesterol transport from perfusate into the wall of human coronary arteries. The significance of the planned work is to investigate mechanisms by means of which this

is brought about. Consequently, although the main thrust of the previous project was directed toward the effect of carbon monoxide, the finding thus obtained now makes possible an investigation of the more general implications of the effect of carbon monoxide on the production of atherosclerosis in human and animal coronary arteries.

For the first time coronary arteries from species other than man can be employed by virtue of the fact that the calculation of the ratio of ^{14}C cholesterol to ^{3}H-cholesterol in the vascular wall makes possible determinations of the amount of cholesterol transported into the coronary arteries of animals regardless of wether or not cholesterol is synthesized. A separation of chemical and hemodynamic factors which may influence cholesterol transfer appears to be of particular importance. Chemical factors responsible for cholesterol transport can be studied by partial destruction of the vascular wall by means of collagenase; the effects thus obtained on cholesterol transfer can be compared with those of hypoxia. The hemodyanmic effects are important because they too, in addition to CO, may play a role in the production of atherosclerosis.

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